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Pancreatic Secretory Trypsin Inhibitor Stimulates the Growth of Rat Pancreatic Carcinoma Cells

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Pancreatic secretory trypsin inhibitor was examined for growth-promoting activity on five cell lines using standard cell culture techniques. One cell line, AR4-2J, derived from a rat pancreatic acinar cell carcinoma, responded with significantly increased incorporation of [³H]thymidine and colony formation. Pancreatic secretory trypsin inhibitor stimulated the incorporation of [³H]thymidine in liquid culture; the maximal increase was $61 \pm 10\%$ above control ($P < 0.001$) and was seen at a concentration of 10^{-9} mol/L. Using a soft agarose clonogenic assay, pancreatic secretory trypsin inhibitor also consistently stimulated (3 assays) colony formation: the peak activity occurred at a concentration of 10^{-10} mol/L which caused a $150 \pm 55\%$ (mean \pm SE, $P < 0.05$) increase above control. Aprotinin had no effect on the growth of AR4-2J cells and pancreatic secretory trypsin inhibitor did not bind to the epidermal growth factor receptor. AR4-2J cells were shown to produce pancreatic secretory trypsin inhibitor. The study raises the possibility that pancreatic secretory trypsin inhibitor provides autocrine stimulation of tumor cell growth.

Pancreatic secretory trypsin inhibitor (PSTI) is a small protein containing 56 amino acid residues that was first isolated from bovine pancreas in 1948 (1) and has since been found in every vertebrate that has been studied. Pancreatic secretory trypsin inhibitor is present in the pancreatic acinar cells and is secreted into pancreatic juice where it is believed to protect against prematurely activated proteases.

The possibility that PSTI may be a growth factor was raised when it was discovered that PSTI has some sequence homology with epidermal growth factor (EGF) and that their corresponding genes share approximately 50% homology (2,3), suggesting a common

ancestry. Pancreatic secretory trypsin inhibitor has already been shown to stimulate the growth of nonneoplastic cells. Rat PSTI increases the growth of mouse 3T3 fibroblasts (4) and human PSTI stimulates the growth of human fibroblasts (5) and endothelial cells (6).

Pancreatic secretory trypsin inhibitor has recently been identified in many extrapancreatic tissues, including the epithelia of the stomach, colon, and small intestine as well as in the liver, lung, kidney, and ovary (7-10). In addition, PSTI has been found to be present in tumors of the ovary (11,12), uterus (12), and lung (13) and cell lines derived from tumors of the liver (14) and pancreas (15). Moreover, PSTI has been shown to bind specifically to a number of cell lines including two derived from colon and epidermoid tumors (16). These findings raised the possibility that PSTI plays a role in stimulating the growth of tumor cells.

Therefore, the effect of PSTI on the growth of four tumor cell lines was examined: three, derived from gastric, pancreatic, and colonic tumors, were chosen because PSTI is normally present in these tissues (17). A vulval epidermoid carcinoma cell line was studied because of the association between PSTI and gynecological tumors. Mouse 3T3 fibroblasts were studied because they had been reported by others to respond to rat PSTI (4). This study shows, for the first time, that human PSTI stimulates thymidine uptake and colony formation in the rat pancreatic acinar carcinoma cell line, AR4-2J, and that this cell line releases PSTI.

Abbreviations used in this paper: EGF, epidermal growth factor; PSTI, pancreatic secretory trypsin inhibitor.

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Materials and Methods

All reagents for cell culture were obtained from Gibco (Paisley, Renfrewshire, Scotland) and chemicals were obtained from BDH (Poole, Dorset, England) unless otherwise stated. Human EGF (urogastrone) was kindly supplied by Dr. Harry Gregory (Bioscience 1; ICI Pharmaceuticals, Macclesfield, Cheshire, England). The sources of the cell lines were MKN45 human gastric carcinoma, D. Morris, Department of Surgery, Nottingham University, Nottinghamshire, England; MC26 mouse colon carcinoma, Dr. Giovana Blaconi, Instituto Marionegri, Milano, Italia; NIH 3T3 mouse fibroblasts, Dr. Natalie Tyke, Imperial Cancer Research Fund, London, England; AR4-2J rat pancreatic carcinoma, European Collection of Animal Cells in Culture, Porton Down, Wiltshire, England, and A431 human vulval epidermoid carcinoma, Dr. Michael Waterfield, Imperial Cancer Research Fund, London, England.

Purification of Pancreatic Secretory Trypsin Inhibitor

Purification of PSTI from human pancreatic juice was based on the method of Iwai et al. (18). Briefly, pancreatic juice not required for clinical investigation was collected from patients who had undergone external pancreatic diversion for clinical reasons. The juice was mixed with an equal volume of sodium citrate, and the pH adjusted to 2.5. Sodium chloride was then added to a final concentration of 1 mol/L and the mixture was maintained at 80°C for 40 minutes, centrifuged at 3500g for 45 minutes at 4°C, and the supernatant concentrated on a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA) equilibrated with 0.05% vol/vol trifluoroacetic acid in water. The cartridge was then eluted with 80% acetonitrile in 0.05% trifluoroacetic acid, and the eluent lyophilized. The eluent was reconstituted in 0.05 mol/L ammonium bicarbonate, pH 8.1, applied to a 1.5 × 100 cm Sephadex G-50 superfine column (Pharmacia, Uppsala, Sweden) and eluted with the same buffer. Fractions containing trypsin inhibitor activity were pooled, lyophilized, and further purified by reverse-phase high-pressure liquid chromatography on a 10 × 100 mm Dynamax C-8 column (12 μm, 300 Å; Rainin, Woburn, MA) eluted with a gradient of 16%–30% acetonitrile in 0.1% trifluoroacetic acid. Separation of PSTI into its various forms was achieved by chromatography on a Mono S column (Pharmacia) equilibrated with ammonium acetate 0.1 mol/L, pH 3.5, and eluted with a gradient of ammonium acetate 0.1 mol/L, pH 3.5–4.5. Of three peaks of trypsin inhibitor activity which emerged, peaks I and III were single peaks when rerun on reversed-phase high-pressure liquid chromatography, whereas peak II separated into 2 peaks; II₁ and II₂. The molecular mass of the four peaks, believed to differ only in the ratio of their asparagine/aspartic acid content (19), was determined by a ZAB-SE mass spectrometer (VG Instruments, Altrincham, Cheshire, England). The molecular masses were I, 6242.5; II₁, 6241.8; II₂, 6241.6; III, 6242.5, compared with the predicted molecular mass for protonated PSTI of 6242.1. Amino acid-sequence analysis of peak I by a protein sequencer (Model 470; Applied Biosystems, Foster

City, CA) showed that the N-terminal tridecapeptide sequence of peak I was equal to that of human PSTI.

Cell Culture

The growth factor activity of PSTI was measured using two standard cell culture techniques, a liquid culture mitogenic assay, and a soft agarose clonogenic assay. Initially, PSTI was screened for growth factor activity using a liquid culture mitogenic assay by growing the following five cell lines in the presence of PSTI at different concentrations: MKN45 human gastric carcinoma, MC26 mouse colon carcinoma, A431 human vulval epidermoid carcinoma, 3T3 mouse fibroblasts, and AR4-2J rat pancreatic acinar cell carcinoma. Stimulation of growth was measured as an increase in the amount [³H]thymidine incorporated into cells. Based on the results, further work on the effect of PSTI on AR4-2J cells was performed using a soft agarose clonogenic assay, in which the stimulation of growth is measured as an increase in the number of cell colonies formed. In addition, both assay techniques were used to examine the effect of aprotinin on the growth of AR4-2J cells. Gastrin (Bachem, Saffron Walden, Essex, England) was used as a control in both the liquid mitogenic assay and the soft agarose assay, except for the studies of 3T3 fibroblasts where EGF was also used. In studies of the effect of aprotinin on the growth AR4-2J cells, EGF and 10% fetal calf serum were used as controls.

Maintenance and Source of Cells

Stock cultures of all cell lines were maintained at 37°C in 95% air, 5% CO₂ in normal growth medium which was RPMI 1640 (Gibco) containing 10% fetal calf serum and 1 mmol/L L-glutamine. They were maintained subconfluent by subculturing two or three times weekly using 0.05% trypsin (2.5% stock solution) and 0.02% ethylenediaminetetraacetic acid.

Liquid Culture Mitogenic Assay

All cell lines were grown as a monolayer in liquid culture in the presence of PSTI (10⁻⁸–10⁻¹³ mol/L or 10⁻⁷–10⁻¹² mol/L). Gastrin (10⁻⁸ mol/L) was used as a positive control in all assays except for those using 3T3 fibroblasts, where both gastrin (10⁻⁸ mol/L) and EGF (3 × 10⁻¹⁰ mol/L) were used as controls. In studies of AR4-2J cells in the presence of aprotinin (10⁻⁷–10⁻¹² mol/L), EGF (3 × 10⁻¹⁰ mol/L) and 10% fetal calf serum were used as positive controls. Each test condition was repeated in six wells and the negative control in 12 wells.

Cells were seeded into 96-well plates (Costar, Sloterweg, Badhoevedorp, Holland) typically at a concentration of 5 × 10⁴ per well in normal growth medium. Plates were then incubated in plastic boxes at 37°C for 72 hours in an atmosphere of 95% air and 5% CO₂. At the end of this period the medium was removed and the test compounds and 1 μCi of [³H]methyl thymidine (5 Ci/mmol/L; Amersham, Aylesbury, Buckinghamshire, England) were added, both were

made up in 100 μ L of serum-free medium. Plates were then incubated for a further 48 hours under the same conditions. The medium was removed from the wells by tipping onto absorbent paper and the cells trypsinized (0.1%) for 30 minutes at 37°C. To each well, 150 μ L of medium was added and triturated to mix. Cells were harvested using a Titertek 530 cell harvester (Flow Laboratories, Rickmansworth, Hertfordshire, England) and the filter papers were allowed to dry. The filter papers were placed into miniscintillation vials together with 2 mL of HP Ready Solve scintillation fluid (Beckman, High Wycombe, Buckinghamshire, England) and counted on an Intertechnique PG-4000 scintillation counter (Kontron Instruments, Watford, Hertfordshire, England).

Soft Agarose Clonogenic Assay

Based on the results obtained from the liquid culture mitogenic assay, further experiments on the growth factor activity of PSTI were performed using a soft agarose clonogenic assay. A base layer was prepared in a 35-mm plastic Petri dish (Costar) by pipetting 1-mL aliquots of 1% Sea Plaque agarose (FMC Bioproducts, Rockland, ME) in normal growth medium. The dishes were poured and the base layers allowed to set for 10 minutes at 4°C. Before assay, the cell suspension was passed through a spinal tap needle two or three times so as to obtain a single cell suspension. The cells were then used quickly to prevent aggregation and dishes were seeded at two different densities to allow for errors in calculating cell density. Final cell count was typically 2×10^4 per dish.

The top layer of the dishes were prepared from 5 mL of 1% agarose, 2.5 mL cell suspension, and 2.5 mL of test compound so as to give the required final concentration. Each test condition was repeated on four dishes and the control on 12 dishes. The assay was used to test PSTI (10^{-7} – 10^{-12} mol/L) and aprotinin (10^{-7} – 10^{-12} mol/L). Gastrin (10^{-10} mol/L) was used as a positive control. Petri dishes were incubated for 10 days at 37°C and the colonies counted using a 40-10 image analyzer (AMS, Saffron Walden, Essex, England).

Pancreatic Secretory Trypsin Inhibitor/Epidermal Growth Factor Binding Studies

AR4-2J cells were maintained in normal growth medium before seeding out into 24 well plates (Costar) at 1×10^6 per well. The plates were incubated for 2 days at 37°C and just before incubation with radiolabel, the cells were washed twice with assay buffer [Earles balanced salts (Sigma, Poole, Dorset, England), 50 mmol/L HEPES, 4.5 mL 22% bovine serum albumin/L (Ortho Diagnostics, Raritan, NJ), 10 mL aprotinin/L, pH 7.0]. Incubation was with 0.1 nmol/L 125 I-EGF (Amersham) alone or in the presence of unlabeled EGF (10^{-6} mol/L) or PSTI (10^{-11} – 10^{-8} mol/L) in assay buffer for 45 minutes at 25°C. At the end of the incubation period the cells were washed three times with 1 mL of cold phosphate-buffered saline, lysed with 1 mL of NaOH (0.1 mol/L), Na_2CO_3 (0.2 mol/L), sodium dodecyl sulphate (35 mmol/L), and 900 μ L of the suspension counted

on a gamma counter (LKB 1272 Clinigamma; Pharmacia, Milton Keynes, Buckinghamshire, England).

Examination of AR4-2J, A431, and MKN45 Culture Media for Pancreatic Secretory Trypsin Inhibitor

Culture medium in which AR4-2J cells had been grown and the other unused mediums (650 mL of each) were centrifuged at 2500g for 30 minutes at 4°C and the supernatant was extracted in the same manner as human pancreatic juice to the point of being concentrated on a C18 Sep-Pak cartridge (Waters, Watford, Hertfordshire, England) and lyophilized.

Extraction of rat PSTI was based on the method described by Greene et al. (19). Pancreas glands were collected from recently killed rats, rapidly frozen on solid carbon dioxide, and stored at -20°C until extraction. The pancreas glands were then suspended in 5 volumes (wt/vol) of 0.1 mmol/L diisopropyl phosphofluoridate at 4°C and homogenized in a blender for 2 minutes. The suspension was adjusted to pH 4.5 with 6 mol/L HClO_4 and centrifuged at 2500 g for 45 minutes at 4°C. The sediment was then reextracted and the combined supernatants brought to 70% saturation by the slow addition of 472 g/L of ammonium sulphate at 4°C. After centrifugation at 2500 g, 4°C for 45 minutes, the supernatant was discarded. The precipitate was resuspended in 0.1 mmol/L diisopropyl phosphofluoridate and stored at -20°C . The extract was then applied to a column (5 \times 100 cm) packed with Sephadex G-75 (Pharmacia) previously equilibrated with 0.5 mol/L KCl, 0.1 mol/L ammonium acetate buffer, pH 4.5. Fractions containing trypsin-inhibitor activity were pooled, lyophilized, and stored at -20°C . The inhibitor was further purified on a G-50 column (1.5 \times 100 cm) in 0.05 mol/L ammonium bicarbonate pH 8.1. Fractions containing trypsin-inhibitor activity were pooled and lyophilized.

The Sep-Pak cartridge eluent obtained by extracting the unused and AR4-2J cell culture medium and a small portion of the rat PSTI were reconstituted in 1 mL of 0.1% trifluoroacetic acid, applied to a 10 \times 100 mm Dynamax C-8 column (12 μ m, 300 Å, Rainin), and eluted with 16%–30% acetonitrile in 0.1% trifluoroacetic acid with isocratic elution at 20% acetonitrile. The fractions were lyophilized and tested for trypsin-inhibitor activity using *N*- α -benzoyl-DL-arginine *p*-nitroanilide as a substrate (20).

Growth medium (50 mL) in which A431 and MKN45 cells had been grown, was treated in a similar manner to that described above for the AR4-2J growth medium. However, the Sep-Pak cartridge eluates were reconstituted in 0.5 mL H_2O and 50 μ L was tested in duplicate for PSTI using a specific radioimmunoassay for human PSTI developed at the Hammersmith Hospital (17).

Results

The initial screen of the five test-cell lines using the liquid culture mitogenic assay showed PSTI to stimulate the incorporation of [^3H]thymidine into AR4-2J cells. In one assay (two plates/fraction) exam-

ining the effects of the different chromatographic forms of PSTI on thymidine incorporation into AR4-2J cells, PSTI peak I had more growth-factor activity than the other three peaks (Figure 1). Pancreatic secretory trypsin inhibitor peak I had most effect at a concentration of 10^{-9} mol/L. This produced an increase in the uptake of thymidine by $61 \pm 10\%$ ($P < 0.001$). This effect was similar to the effect of the positive control which was gastrin at 10^{-8} mol/L (Figure 1). The other four cell lines, MKN 45, MC26, A431, and 3T3, showed no response to PSTI (Figure 2). Mouse 3T3 fibroblasts did not respond to either gastrin or PSTI in the liquid culture mitogenic assay but showed 147%–193% increases in thymidine uptake above control in the presence of EGF (3×10^{-10} mol/L).

Soft agarose clonogenic assay showed PSTI peak III to stimulate colony formation consistently in the rat pancreatic acinar cell line, AR4-2J, in 3 assays. Peak activity occurred at 10^{-10} mol/L, (Figure 3) causing a $150 \pm 55\%$ (mean \pm SE, $P < 0.05$) increase in colony formation above control. In this assay, PSTI had slightly more activity than the positive control, gastrin (10^{-10} mol/L). Aprotinin also had no effect on either thymidine incorporation or colony formation by AR4-2J cells when it was added into the culture medium at a concentration range of 10^{-7} – 10^{-12} mol/L (Figure 4).

When AR4-2J cells were incubated with ^{125}I -EGF, 94% of bound tracer could be displaced in the presence of an excess of cold EGF. In the presence of unlabeled PSTI (10^{-8} – 10^{-11} mol/L) no detectable

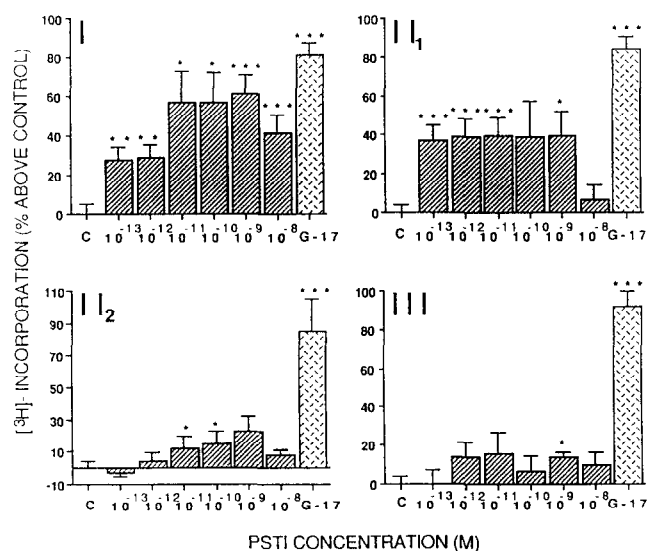


Figure 1. Stimulation of $[^3\text{H}]$ thymidine incorporation into the rat pancreatic carcinoma cell line, AR4-2J, by forms I, II, II₁, and III of human PSTI in liquid culture mitogenic assay. Positive control is gastrin-17 (10^{-8} mol/L). The vertical axis indicates the change in $[^3\text{H}]$ thymidine uptake. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ compared with control.

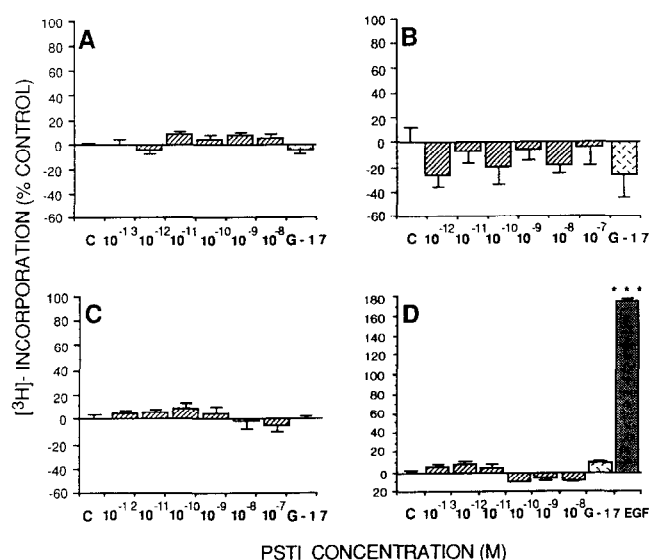


Figure 2. Investigation of possible stimulation of $[^3\text{H}]$ thymidine incorporation into (A) human vulval epidermoid carcinoma cell line, A431, (B) human gastric carcinoma cell line, MKN45, (C) mouse colon carcinoma cell line, MC26, and (D) 3T3 mouse fibroblasts in the presence of PSTI in a liquid culture mitogenic assay. Gastrin-17 (10^{-8} mol/L) was used as a control in each study and in (D) EGF (3×10^{-10} mol/L) was also used as a control. The vertical axis indicates the change in $[^3\text{H}]$ thymidine uptake. *** $P < 0.001$ compared with control.

inhibition of the binding of the ^{125}I -EGF was observed (Figure 5).

Analysis of the culture medium extracts on reverse-phase, high-pressure liquid chromatography showed a peak of trypsin-inhibitor activity that was present in the medium conditioned by AR4-2J cells but not in the unused medium extract. The peak of trypsin-inhibitor activity eluted in about the same position as rat pancreatic PSTI (Figure 6). Analysis of the culture medium in which A431 and MKN45 cells had been

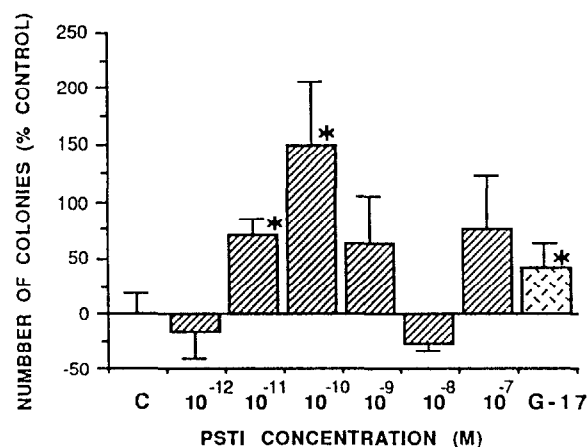


Figure 3. Stimulation of colony formation of the rat pancreatic carcinoma cell line, AR4-2J, by PSTI (peak III) in soft agarose assay. Positive control was gastrin-17 (10^{-10} mol/L). * $P < 0.05$ compared with control.

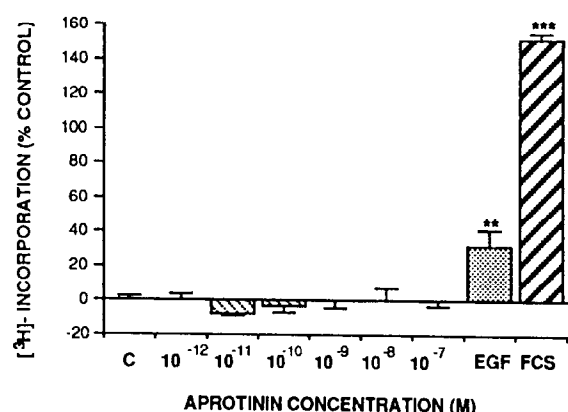


Figure 4. The effect on [³H]thymidine incorporation in the rat pancreatic carcinoma cell line, AR4-2J, grown in the presence of aprotinin in liquid culture mitogenic assay. Positive controls were EGF (3×10^{-10} mol/L) and 10% fetal calf serum. **P < 0.01, ***P < 0.001 compared with the control.

grown showed the extract to contain no detectable PSTI-like immunoreactivity (<20 ng/L of medium).

Discussion

In this study we have shown that human PSTI stimulates the growth of AR4-2J cells which are derived from a rat pancreatic acinar carcinoma cell line. We have also demonstrated that these cells release PSTI. These two findings raise the possibility that PSTI may be an autocrine stimulant of tumor-cell growth.

This study is the first to show that PSTI can stimulate the growth of neoplastic cells. Earlier studies have shown that one of the two forms of rat PSTI, monitor peptide, stimulates thymidine and methionine incorporation as well as increases the cell number of 3T3 mouse fibroblasts in liquid culture (4). However, we were unable to demonstrate an effect of human PSTI on the incorporation of thymidine into these cells

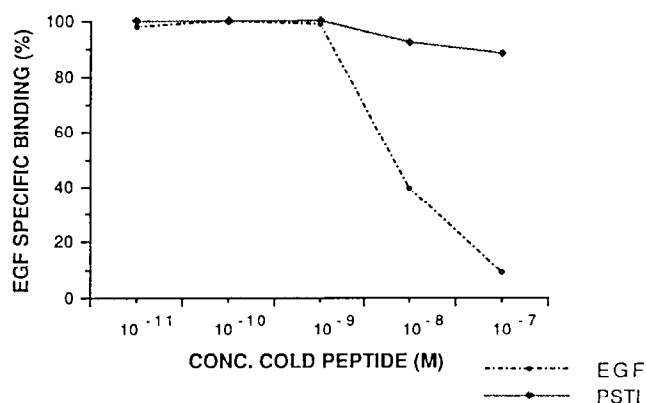


Figure 5. The binding of ¹²⁵I-EGF (percent specific binding) to AR4-2J rat pancreatic carcinoma cells in the presence of different concentrations of PSTI.

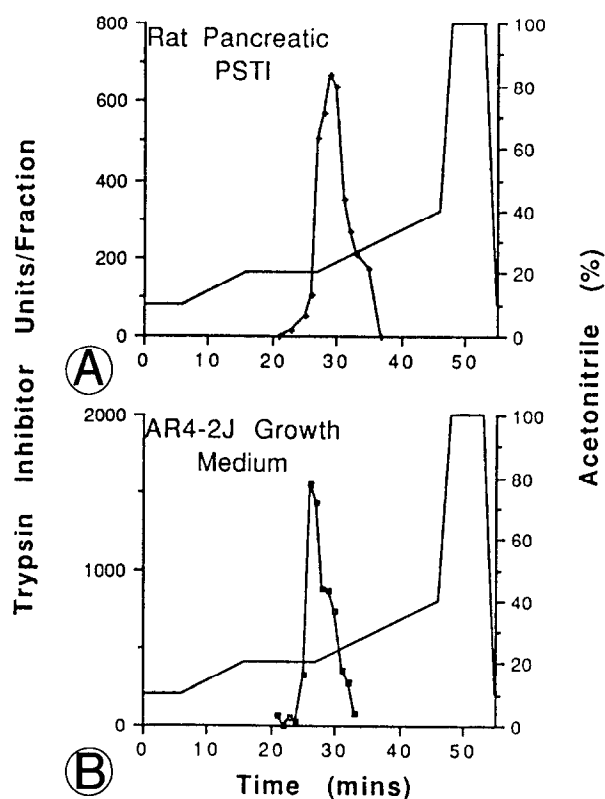


Figure 6. The elution profile of trypsin-inhibitor activity extracted from (A) rat pancreas and (B) growth medium in which AR4-2J cells had been grown from reverse-phase, high-pressure liquid chromatography (Dynamax, C-8, 10 × 100 mm, 12 μm, 300 Å). (—■—) Trypsin-inhibitor units/fraction, (—) % acetonitrile.

under the conditions that we used. Human PSTI has been shown to stimulate the uptake of thymidine into human fibroblasts (5) and to stimulate the growth of human endothelial cells (6).

There are similarities between PSTI and EGF and, at present, there is controversy as to whether PSTI stimulates growth by interacting with EGF receptors or other receptors. The human peptides are of a similar size, PSTI containing 56 amino acid residues and EGF containing 53: both have three disulfide bridges (21). Analysis of their respective genes shows about 50% homology, suggesting a common ancestral origin (2). Also, the results of binding studies suggest that rat PSTI competes with EGF for binding to receptors on 3T3 mouse fibroblasts (22). However, only 7 of the 56 amino acid residues comprising human PSTI are homologous with those of human EGF (3). Niinobu et al. (16) examined the binding of PSTI to nine cell lines and found human PSTI to bind to eight of the cell lines including five fibroblast lines, an endothelial cell line, and two tumor cell lines derived from an epidermoid and colon carcinoma. However, the binding of PSTI to the cell lines did not correlate with the binding of EGF to the same cell lines, suggesting in this case that PSTI was binding to a

different receptor. Our results support this view. Out of the five cell lines tested in this study, 3T3, MKN45, and AR4-2J were EGF responsive in our hands but only one of these, the AR4-2J cell line, was stimulated by PSTI. This was demonstrated in the liquid culture mitogenic assay of mouse 3T3 fibroblasts which responded well to EGF but not to PSTI. In addition, our studies demonstrated that the binding of ^{125}I -EGF to AR4-2J cells was unaffected in a 100-fold excess of cold PSTI. It now seems important to characterize the PSTI binding site that is present on normal and neoplastic cells (16). In view of the differences in the growth-stimulating effects of the different forms of human PSTI shown in this study, it seems likely that deamidation of PSTI affects its binding to such sites.

McKeehan et al. found that the growth of human endothelial cells was stimulated by the trypsin inhibitors aprotinin and α_1 -antitrypsin as well as PSTI (6). This could reflect the similarities between inhibitor/enzymes and growth-factor/receptor interactions, perhaps reflecting an ancient common origin. Alternatively, the trypsin inhibitors could protect other growth factors from extracellular proteases as cultured endothelial cells exhibit significant proteolytic activity. However, unlike PSTI, aprotinin did not stimulate the growth of AR4-2J cells.

AR4-2J cells secrete amylase and trypsinogen, and in the normal pancreas these are cosecreted with PSTI. Therefore, our chromatographic demonstration that AR4-2J cells produce PSTI confirmed what was expected but had not been demonstrated previously. It is of particular interest that the human pancreatic cancer cell line CAPAN-1 also produces large amounts of PSTI (15) and that three other human pancreatic cell lines show PSTI immunoreactivity (23). Rat PSTI has been fully characterized by others and found to comprise two discrete forms. Rat PSTI-I contains 61 amino acid residues and possesses cholecystokinin-releasing activity and has been called monitor peptide. Rat PSTI-II has 71% homology with rat PSTI-I and 63% homology with human PSTI and, like human PSTI, contains 56 amino acid residues (24).

Malignant tumors of a variety of organs are often accompanied by increased serum and urine concentrations of PSTI (8,23,25,26) which, before being identified as PSTI (27), was known as tumor-associated trypsin inhibitor. Extrapancreatic human tumors also produce PSTI. A hepatoblastoma cell line (14) secreted PSTI, and PSTI was found to be present in cell lines derived from gastric cervical cancers (23) and a hepatoma (6). Pancreatic secretory trypsin inhibitor has been identified in ovarian (11,12) and cervical cancers (12) and messenger RNA encoding PSTI was present in a pulmonary adenocarcinoma and in a colonic polyp (13).

The stimulation of the growth of AR4-2J cells by

PSTI which is itself secreted by these cells, raises the important possibility that PSTI acts as an autocrine stimulant of neoplastic growth. Further studies will examine the effect of specific immunoneutralization of PSTI on tumor-cell growth. The findings of others suggest that such studies should be extended to tumors other than those of the pancreas.

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